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Effects of Vitamin Antimetabolites on *Lebistes reticulatus.*

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(Text-figures 1 & 2)

INTRODUCTION

THE role of vitamins in the nutrition of fishes poses a complex problem both to workers in the field of pure nutrition and to fish culturists. Consequently, there has been a lack of extensive research, as indicated by the scarcity of reports in the literature, on the nutrition of fishes other than trout. Most of the studies in the past have dealt with the relationship between various combinations of different amounts of natural foods and their effects on the growth rate of fishes. Early studies provided no information about the chemical components necessary for normal growth. Embody & Gordon (1924) reported on the natural and artificial food of trout. More recent work by Wolf (1951), Halver & Coates (1957), Halver (1957) and Coates & Halver (1958) has indicated some success in composing synthetic diets based on the requirements of Embody and Gordon.

The varied composition of natural foods, with necessary growth factors and trace elements, makes it unlikely that fishes in nature, both fresh and salt water, are often afflicted with dietary deficiencies. Comfort (1956) stated that the weight of evidence suggested that senescene in the wild is rare but not unknown.

On the other hand, it has been known for a long time that fishes in captivity, mainly those raised by government-controlled hatcheries, are susceptible to various pathological conditions caused in many cases by the use of synthetic diets (Wolf, 1951). The use of synthetic diets lies in the importance of the artificial breeding of these fish in large numbers at low cost. Very little work has been done on marine fishes, freshwater fishes of no interest to anglers and on "tropical fish" found in aquaria. Although synthetic diets for salmon (Halver, 1957) and trout (Wolf, 1951) have been reported, these large cold water salmonoid fishes do not lend themselves readily to laboratory experimentation under controlled conditions.

The use of vitamin antimetabolites is a convenient method to study vitamin deficiencies in an organism where a satisfactory synthetic diet has not been formulated. Groups of fish were also reared under axenic conditions, thus improving environmental control to a considerable degree. A further objective of this study was the effect of the antivitamins on the growth and mortality of the guppy *(Lebistes reticulatus).* There has been no report in the English literature of a normal growth curve (weight plotted against time) for any warm water "tropical fish." Such growth curves, including one plotting length against time, and growth curves resulting from the effects of the analogs were determined in this study.

Some of the basic concepts of the action of antimetabolites stem back to the time of Paul Ehrlich (1907) who coined the term "chemotherapy". The idea of competitive inhibition had its roots in the work of Michaelis & Menten (1913) and later (1927) in the work of Quastel & Wooldridge who showed the competitive inhibition of succinic dehydrogenase by malonic acid, a structural analog of succinic acid. Following the report of Woods (1940) on the action of sulfanilamide, Fildes (1940) proposed a rational approach to chemotherapy by the use of

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structural analogs of known essential metabolites.

Thiamine is required by most living organisms. Phillips, *et al,* (1946) produced the first critical work on fishes in establishing the thiamine requirement of trout. The presence of a thiamine-splitting enzyme in nature was first reported to be found in carp viscera which were fed to foxes (Green & Shillinger, 1936; Green, Evans & Carlson, 1937). These foxes developed a typical polyneuritic sympton that was referred to as "Chastek paralysis." This condition was relieved by the administration of thiamine. Wooley (1941) found that carp tissue contained a thiamine-splitting enzyme that was thermolabile and nondialyzable. Wolf (1942), while working on trout, noted thiamine deficiency symptoms when diets containing raw fish were used. This thiamine-destroying principle was subsequently called thiaminase. Deutsch & Hasler (1943) studied the distribution of thiaminase among freshwater fishes while Yudkin (1945) investigated the occurrence of thiaminase in marine teleosts. Thiaminase from whole carp and fractions elicited deficiency in chicks (Spitzer, Coombes, Elvehjem & Wesnicky, 1941), and feeding on carp eggs caused avitaminosis and death to the catfish *Schilbeodes mollis* (Harrington, 1954).

The bracken fern *Pteris aquilina* appears to be another source of a thiamine-destroying principle. Horses and cattle which had consumed large amounts of bracken fern became ill with "fern poisoning" (Weswig, Freed & Haag, 1946). Recent reports indicate a mass poisoning of calves by *Pteris aquilina* (Gregorovic, Skusek & Senk, 1962).

Deoxypyridoxine effects have been studied in the chick (Ott, 1946), chick embryo (Cravens & Snell, 1949; Karnofsky, *et al.,* 1950), rat and mouse (Umbreit, 1955). The work on fishes with the metabolite pyridoxine has been less critical than that on higher vertebrates. In trout, its absence, plus the absence of riboflavin and pantothenic acid, were collectively believed to cause anemia (Tunison *et al.,* 1944). McLaren, *et al.,* (1947) working with purified rations to produce pyridoxine deficiency on the trout, *Salmo gairdneri,* reported nervous disorders, epileptiform fits and light spots on the liver. Halver (1953) working with vitamin-free basal rations on the chinook salmon, *Onchorhynchus tshawytscha,* reported pyridoxine deficiency symptoms such as nervous disorders, epileptiform fits, hyperirritability, ataxia, anemia, anorexia, edema of the peritoneal cavity, colorless serous fluid, spastic convulsions, blue coloration on back, rapid and gasping breathing, flexing of the opercles and *post mortem rigor mortis* occurring rapidly.

Biotin has been referred to as the "anti egg-white injury factor" (Lease & Parsons, 1934) and is found almost universally in plants and animals. A deficiency caused by feeding egg white containing avidin to rats elicited dermatitis, retarded growth, loss of hair and muscular control (Martin, 1951). The first antimetabolite of biotin synthesized was desthiobiothin which was active on *Lactobacillus casei* (duVigneaud, 1942). Phillips, *et al.,* (1947) established the dietary need for biotin in trout. Phillips, Brockway & Rodgers (1950) reported that a dietary biotin deficiency in brown trout caused a condition characterized by a bluish film covering the body. This coating eventually sloughed off giving the trout a patched appearance. The disease was referred to as "blue-slime" or "slime-patch." McLaren, *et al.,* (1947) reported that biotin deficiency caused anorexia and retarded growth in trout and Halver (1953) noted that a deficiency in salmon caused a dark coloration, muscle atrophy, spastic convulsions and fragmentation of erythrocytes.

The characteristic syndrome of ascorbic acid (vitamin C) deficiency or scurvy has been recognized for centuries. Woolley & Krampitz (1943) reported on the first ascorbic acid analog, glucoascorbic acid. They produced a syndrome in rats and mice induced by glucoascorbic acid somewhat paralleling that of scurvy, even though rats and mice do not ordinarily require this metabolite. However, Wooley (1944) soon demonstrated the antivitamin action of glucoascorbic acid on guinea pigs, which do require ascorbic acid. In the field of fish nutrition, McLaren, *et al.,* (1947) reported nutritional deficiency symptoms in trout, while Wolf (1951) and Halver (1953) reported that ascorbic acid was not necessary in trout and in salmon, respectively.

Materials and Methods

The following antimetabolites were used: oxythiamine (OB_1), in which the amino group in position 4 of the pyridine moiety of thiamine was replaced by a hydroxyl group, (Text-fig. 1), (Bergel & Todd, 1937); neopyrithiamine (NPT) or purified pyrithiamine, formed by the displacement of the thiozole nucleus with a pyrimidine ring, (Text-fig. 1) (Wilson & Harris, 1949); thermolabile factor (LF) and thermostabile factor (SF), extracted from the fern *Pteris aquilina* by cold acetone, (Fujita, 1954); aqueous labile factor (ALF) and aqueous stabile factor (ASF) extracted from *P. aquilina;* desthiobiotin (DB), in which the tetrahydrothiophene ring of biotin was

split and the sulfur atom eliminated, (duVigneaud, 1942); deoxypyridoxine (DB_6), in which there was a replacement of the hydroxymethyl group of pyridoxine by a methyl group at position 4, (Ott, 1946), and glucoascorbic acid (GAA), a 7 carbon analog of ascorbic acid (Woolley & Krampitz, 1943).

Newborn guppies *(Lebistes reticulatus)* of unknown genetic stock and raised in the investigator's laboratory were used throughout the experiment. Single litters were chosen for the procedure outlined.

Non Axenic Conditions

The young fish in groups of 10 were placed in 200ml of "conditioned" boiled aquarium water of pH 7.2 at a temperature of 23.0°C. "Conditioned" aquarium water is water in which fish previously had lived (Allee, 1938). Round, stacked culture dishes were used as containers in the non-axenic controls and experimental groups with the added antimetabolite. The analogs were added directly to the water. The fish were measured with a caliper, weighed (wet) and transferred weekly to water with fresh concentrations of antimetabolites.

Preparation of Extracts and Diet

The concentrations in micrograms of antimetabolites employed were as follows: 1, 2, 3, 4, 5, 10, 20, 40, oxythiamine; 5, 10, 25, 50, 100, pyrithiamine; 50, 100, 200, desthiobiotin; 5, 50, 100, deoxypyridoxine; 50, 100, 250, glucoascorbic acid; and in percent solution: 1, 2, 5, 10, 15, aqueous non-heated fern extract (ALF), (50gm triturated leaves per liter of distilled water and filtered after standing for 30 minutes); 1, 2, 5, 10, 15, aqueous heated fern extract (ASF), (heated to boiling for 10 minutes); and in mg percent: 0.5, 5, 10, 40, of cold acetone extracted precipitate from fern (LF); and 5, 10, 20, 40, of powder from evaporated fern filtrate (SF). The analogs were obtained from commercial and private sources, while the fern extract (LF and SF) were prepared by the method of Fujita (1954).

The experiments were conducted over a period of 12 weeks so that sufficient time would be available for sexual differentiation.

The diet consisted of a modification of the liver-cereal wet food and standard dried food of Gordon (1950). The fish were fed three times a week.

Axenic Technique

The procedures previously outlined were repeated with modification, using axenic fish as follows:

Gravid females in groups of two were placed in water for 48 hours containing 50mg of chlortetracycline HCl per liter as a preparation for obtaining germfree young. Wendt (1956) demonstrated that 5.0mg% chlortetracycline HCl produced no statistical significance on the weights and lengths of guppies as compared to that of controls at the 15-week stage. During the 48 hours of preparation time, no food was administered in order to clear the intestinal tract, since it had been previously reported that fasting fish normally do not have bacteria in their intestinal tract and that the organisms are introduced only at the time of food intake (Margolis, 1953). The fish were placed in chlorobutanol anesthesia solution until complete immobilization was observed, and this was followed by a three-minute immersion in tincture of merthiolate. They were passed through two washings of 70 percent alcohol and placed on sterile gauze. With sterile instruments, an incision was made between the anal opening and anal fin at about a 45-degree angle, tangentially to the peritoneal cavity. The peritoneum was thus left temporarily intact and the operating area remained sterile. An opening was then made in the silvery peritoneum and the ovarian membrane was ruptured. A gradual pressure on the branchial region of the fish caused the entire clump of embryos to protrude. The embryos were then dropped in sterile distilled conditioned water in a syracuse crystal placed in a petri dish. The water contained salts in the following concentrations: 0.8% NaCl; 0.024% $CaCl_2$ 0.042% KCl; 0.1% $NaHCO_3$. In order to prepare this medium, the salts were dissolved in distilled water and guppies were placed in it for 24 hours. 200ml of this "conditioned fish saline" were placed in 500ml cotton-stoppered flasks and autoclaved. The embryos obtained by sterile technique were placed in this conditioned fish saline in groups of 10. Autoclaved fish food was introduced three times a week. Oxythiamine, pyrithiamine, desthiobiotin, glucoascorbic acid and deoxypyridoxine in the minimum concentrations were passed through a Seitz bacterial filter and introduced into the sterile cultures. Every 24 hours after feeding, one ml of water was removed and introduced into an agar plate to test sterility.

In order to establish the effective dosage for each analog in the non-axenic groups, preliminary experiments of the immersion type were conducted in which the concentration of the analog was increased until atypical behavior of the fish was observed or the maximum solubility point of the analog was reached.

Reversal

One of the criteria for confirming the status of a substance as an antimetabolite in an organism has been the ability of the respective metabolite to reverse the effects of the analog. Because of a significant difference in weight and length between male and female guppies, in the first reversal experiment only adult male guppies in groups of six were tested. Fish in groups of three were fed on alternate days 50mg of liver-cereal food which also contained 10% analog by weight. This was necessary in order to inactivate the vitamin in the food, so that continued feeding would not introduce an excess of metabolite which would raise the required amount of antimetabolite necessary to cause inhibition.

When a 50% mortality of the fish occurred, the respective metabolite was added to the solution. The average weight of the groups of fish was recorded and the survivors were weighed in four days.

Reversal in young immature fish was demonstrated in the following manner:

Week-old guppies in groups of 12 were placed in 200ml of conditioned water. In addition to a control group, there were five groups, each of which contained antimetabolites in a solution containing oxythiamine, 40μg; pyrithiamine, 100μg; aqueous non-heated fern extract (ALF), 1%; acetone extracted fern (SF) 40mg%; and deoxypyridoxine, 100μg. Every two days each group was fed 50mg of liver-cereal wet food, containing 10% of the respective antimetabolite used in solution. The purpose of the last procedure was to inactivate the natural vitamins in the food by the 10% antimetabolite portion, thus allowing the antimetabolite in solution to act directly upon the fish. When a minimum of ⅓ mortality was reached for each group the reversal phase of the experiment was initiated.

Corresponding metabolite was added to each group at an equivalent or greater concentration than the original concentration of the antimetabolite. Subsequent feedings of 50mg of liver-cereal wet food were continued on alternate days but without the addition of any metabolite.

The average weight of each group was determined at the onset of the experiment and, thereafter, at seven-day intervals. The data in Text-figure 2 were carried up to seven weeks growth since it is possible that the length and especially the weight of the fish may be unduly influenced by the male differentiating at seven weeks. This results in a relative stabilization of male weights while female weights will continue to rise. The presence of a greater number of females than males after seven weeks of growth results in a sharper growth curve.

Results

Normal Growth

Growth was measured as the average mean weight and average mean standard length (snout to caudal peduncle). The data indicate that the weight rose from 8.7 mg at the end of the first week to 18.5 mg by the end of the 7th week (Text-fig. 2, A & D). A marked increase in weight was observed between the first and second weeks. The weight increase after the second week and up to the seventh week was gradual. The rate of growth measured in terms of length did not show a gradual weekly increase but was variable and indicated the lower part of a sigmoid curve. At seven weeks of age, sexual differentiation of the males was observed, characterized by the development of the typical male pigmentation and gonopodium. Sexual differentiation of the females occurred by the 12th week.

Oxythiamine

Oxythiamine was effective in eliciting thiamine deficiency symptoms in a minimal concentration of 1 μg, resulting in a survival period of 16.2 days. The survival period at a maximum concentration of 40 μg was reduced to 3.7 days. However, no effects were observed before three days, regardless of the concentration of analog used. The effects of oxythiamine on the weight and length of the guppy indicate a weight increase up to the fourth week followed by a sharp drop in the fifth week (Text-fig. 2A). The effect on length was minimal. The onset of the deficiency syndrome similar to that induced by thiamine nutritional deficiency as described by Halver (1953, 1957) was characterized by a general loss of equilibrium. The fish swam or remained motionless on their sides. Eventually they swam in a spiral fashion and often with their heads on the bottom of the container and their bodies vertical. At times, they remained motionless near the surface or close to the bottom. General anorexia was observed after 24 hours. The critical point of the deficiency syndrome was the onset of the spastic convulsive swimming movements, ataxia and rapid flexing of the opercles, followed by periods when the fish was inverted on the bottom with only the pectoral fins in slight motion. Death occurred within 24 hours of the latter symptoms. Oxythiamine appeared to be a more powerful displacer of thiamine than was pyrithiamine, the next analog tested, since a 10 μg concentration of oxythiamine was sufficient to reduce the survival time to a minimal range of

3.9 days, whereas 10 μg of pyrithiamine resulted in a survival period of 27.4 days.

Pyrithiamine

The effect of this analog on survival indicates that it is less active than oxythiamine at a similar concentration (Table 1). However, pyrithiamine has a greater effect on the weight and a comparable effect on the length of the fish when compared to the effects of oxythiamine (Text-fig. 2A).

Thiaminase Fern Extract

The acetone extracted thermolabile portion of the fern extract (LF) elicited no grossly visible effects resembling thiamine deficiency (Table 1). However, the acetone extracted thermostable factor (SF) which was the brick red powder resulting from the vacuum and heat drying of the supernatant material showed activity (Table 1). The aqueous extractions of the fern demonstrated that the aqueous labile factor (ALF) was effective while the aqueous stabile factor (ASF) was not effective in survival (Table 1). Similar effects were noted on the weight of the fish (Text-fig. 2C). Therefore, SF and ALF showed activity while LF and ASF showed no activity.

Desthiobiotin

Desthiobiotin had no adverse effects on the growth of the guppy raised in non-axenic condition (Table 1). There was no evidence of anorexia or blue slime-patch disease. However, when newborn guppies were axenically raised, desthiobiotin proved to be an active antagonist. At 18 days from the start of the experiment, a generalized ataxia was observed, in addition to a darker body coloration. However, there was no sloughing off of any part of the epidermis as is the case in blue slime-patch disease of trout. By 21 days, no fish had survived the experimental treatment.

Deoxypyridoxine

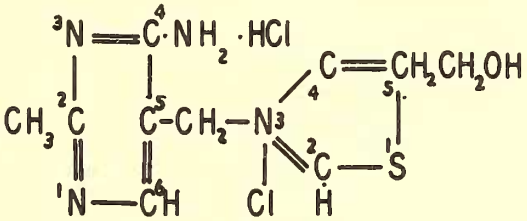
Deoxypyridoxine was an active analog of pyridoxine in the guppy at concentrations of 100 μg (Table 1). In comparing this analog with the others used, it demonstrated a greater effect on inhibiting weight increase, with the result that there was no significant increase in the first six weeks (Text-fig. 2B). In addition, ataxia, anorexia, "yawning" of the mouth and flexing of the opercles were observed.

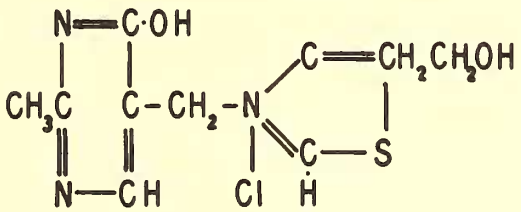
Glucoascorbic Acid

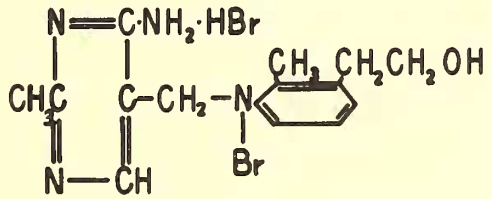
In a maximum concentration of 250 μg of glucoascorbic acid, no abnormal indications of growth, appetite or mortality were observed in the fish raised in non-axenic culture. The fish raised axenically showed retardation of growth, generalized edema and a high mortality with no survivors by the sixth week (Text-fig. 2D).

Reversal

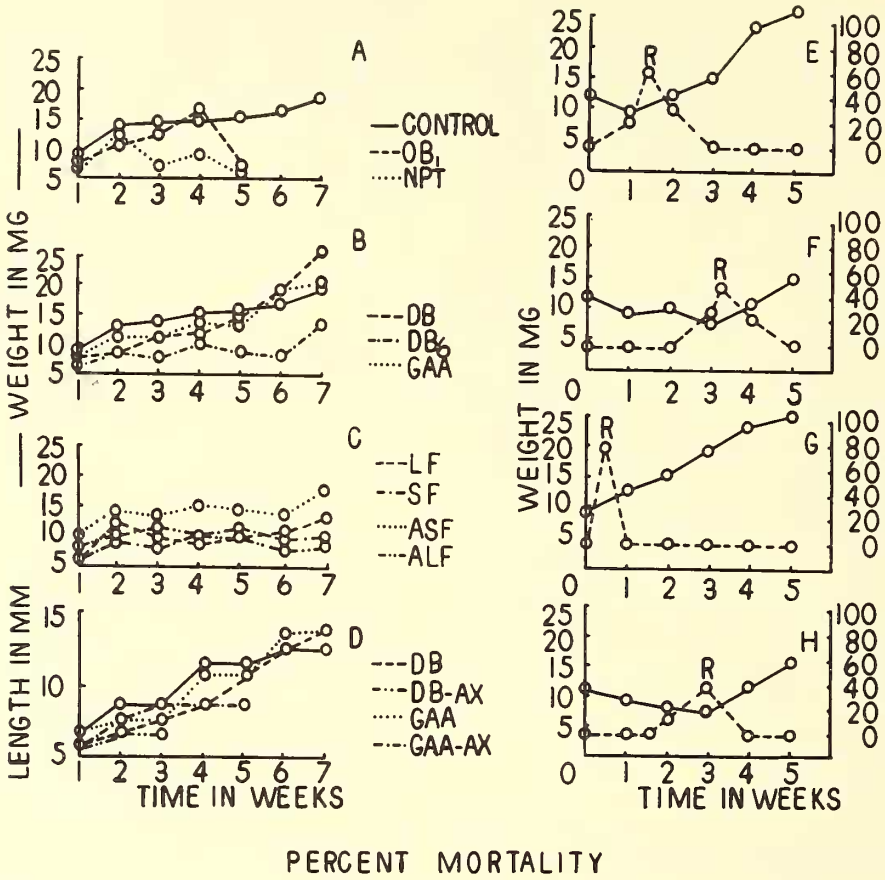
The reversal experiment with adult fish indicated that oxythiamine was more active than pyrithiamine, since oxythiamine caused 50% mortality in fish in a shorter time and in a lesser concentration (Table 2, Text-fig. 2E). When thiamine was added on the 10th day, the per cent mortality decreased and the weight increased. A reversal by the normal metabolite was also noted with pyrithiamine, fern extracts ALF and SF and deoxypyridoxine (Table 3, Text-fig. 2, F, G & H).







TEXT-FIG. 1. Oxythiamine and pyrithiamine, two analogs of thiamine.



TEXT-FIG. 2. A: The effect of oxythiamine (OB_1) and pyrithiamine (NPT) on the weight of *Lebistes;* B: desthiobiotin (DB), deoxypyridoxine (DB_6) and glucoascorbic acid (GAA) effects on weight; C: fern labile factor (LF), fern stable factor (SF), fern aqueous stable factor (ASF) and fern aqueous labile factor (ALF) effects on weight; D: desthiobiotin (DB), desthiobiotin-axenic culture (DB-AX), glucoascorbic acid (GAA) and glucoascorbic acid-axenic culture (GAA-AX) effects on length; E: oxythiamine (OB_1) reversal by thiamine and percent mortality; F: pyrithiamine (NPT) reversal by thiamine; G: fern aqueous labile factor (ALF) reversal by thiamine; H: deoxypyridoxine (DB_6) reversal by pyridoxine.

TABLE 1. THE EFFECT OF ANALOGS ON THE SURVIVAL OF THE GUPPY

(Ten fish used at each concentration.)

Analog	Concentration	Survival in days (30 day period)
Control A	(non-axenic)	30
Control I	(axenic + food)	30
Control II	(axenic no food)	6 ± 0.211
	micrograms	
oxythiamine	1	16.2 ± 3.83
	2	17.7 ± 3.46
	3	8.3 ± 2.47
	4	9.0 ± 3.12
	5	11.2 ± 2.54
	10	3.9 ± 0.74
	20	5.1 ± 0.32
	40	3.7 ± 0.21
pyrithiamine	5	28.1 ± 1.29
	10	27.4 ± 1.42
	25	21.4 ± 2.56
	50	18.0 ± 1.78
	100	11.3 ± 1.82
deoxy-pyridoxine	5	28.4 ± 1.60
	50	28.7 ± 1.30
	100	19.6 ± 1.43
desthiobiotin	50	30.0
	100	30.0
	200	28.4 ± 1.60
	200 (axenic)	19.1 ± 0.32
glucoascorbic acid	50	30.0
	100	30.0
	250	30.0
	250 (axenic)	27.2 ± 1.17
	per cent	
aqueous labile factor (ALF)	1	27.3 ± 2.70
	2	4.2 ± 0.36
	5	1.0
	10	1.0
	15	1.0
aqueous stable factor (ASF)	1	30.0
	2	30.0
	5	27.4 ± 2.70
	10	30.0
	15	30.0
	mg per cent	
labile factor (LF)	0.5	30.0
	5	28.0 ± 2.00
	10	30.0
	40	30.0
stable factor (SF)	5	28.2 ± 1.80
	10	25.5 ± 3.02
	20	30.0
	40	2.6 ± 0.27

TABLE 2. REVERSAL OF ANTIMETABOLITES IN THE ADULT MALE GUPPY *(Lebistes reticulatus)*.

Antimetabolite	Concentration	Number of fish at start	Time to produce 50% mortality (in days)	Initial weight in mg. (avg.)	Weight in mg. at 50% mortality (avg.)	Weight 4 days after start of reversal treament[a] (avg.)	Concentration of metabolite used for reversal	Number of survivors 4 days after time of 50% mortality
control	0	6	0	123	...	130[b]	0	6
oxythiamine	40μg	6	4	131	120	126	40μg	3[c]
pyrithiamine	100μg	6	7	121	111	116	100μg	3
aqueous non-heated fern (ALF)	1%	6	3	126	120	122	4mg%	3[d]
fern thiaminase stable factor (SF)	40mg%	6	4	128	125	126	40mg%	3
deoxypyridoxine	100μg	6	6	108	104	104	100μg	3

a) Reversal treatment was started after LD/50 was reached.
b) Average control weight 10 days after start of experiment.
c) One fish from this group not fully recovered at 4 days after start of reversal.
d) One fish from this group not fully recovered at 4 days after start of reversal.

TABLE 3. REVERSAL OF ANTIMETABOLITES IN THE IMMATURE GUPPY *(Lebistes reticulatus)*.

Antimetabolites	Concentration	Metabolite & concentration	Time (days) required to produce ⅓ mortality[R]	Number of fish at start	Number of fish at reversal time	Average weight in mg. Days 0	7	14	21	28	35	Number of fish at 35 days
control	0	0	0	12	12	14.1	16.5	18.6	20.0	23.7	26.2	12
oxythiamine	40μg	40μg thiamine	10	12	3	11.8	9.6[R]	12.0	15.0	24.0	28.0	2
pyrithiamine	100μg	100μg thiamine	23	12	4	11.5	9.0	9.2	8.8[R]	11.0	14.8	4
aqueous non-heated fern extract (ALF)	1%	4mg% thiamine	3	12	3	9.5[R]	13.0	15.8	22.3	27.0	29.5	3
acetone extracted fern (SF)	40mg%	40mg% thiamine	3	12	4	9.4[R]	10.0	18.2	30.0	32.5	34.1	4
deoxypyridoxine	100μg	100μg pyridoxine	21	12	6	10.7	9.8	9.8	9.5[R]	11.1	16.0	6

R = time that reversal was started.

Table 4. Antimetabolite Syndromes

Analog	Signs of avitaminosis	
	In Lebistes	In other organisms
oxythiamine	Polyneuritic-type symptoms; loss of equilibrium; ataxia; rolling; "standing on head;" anorexia; convulsions and death, survival time 3-4 days.	No polyneuritis, but anorexia, weight loss and death in mice (Soodak and Cerecedo, 1947); increased blood pyruvate and blood lactate in rats, depression of thiamine in tissues, increased thiamine excretion (Frohman and Day, 1949); retardation of growth, head retraction, convulsions and death in the chick (Daniel and Norris, 1949); local and general edema in chick embryo (Naber, *et al.*, 1954); competive inhibition in mice (Cerecedo, et al., 1951).
pyrithiamine	Polyneuritic-type symptoms; ataxia; loss of equilibrium; temporary immobility; scoliosis; anorexia; convulsions and death; survival time 12-28 days.	Polyneuritis, anorexia, weight loss and death in 7-8 days in rats and mice (Eusebi and Cerecedo, 1949); polyneuritis and death in chick embryos (Naber, *et al.*, 1954); normal blood pyruvate and liver cocarboxylase (Woolley and Merefield, 1952); decrease of total thiamine in muscle, liver and brain of rat (DeCaro, *et al.*, 1954); competitive inhibition in bacteria (Woolley and White, 1943) and in mice (Eusebi and Cerecedo, 1949).
aqueous fern extract (ALF)	Loss of equilibrium, ataxia; polyneuritis; convulsions; death in 24 hours in 2% solution.	Polyneuritis, reduced blood thiamine, elevated blood pyruvate in rats (Evans and Evans, 1949); same in horses (Evans, *et al.*, 1951), but not in cattle (Evans, *et al.*, 1954).
aqueous heated fern extract (ASF)	No effect	Factor is labile (Thomas and Walker, 1949; Evans and Jones, 1952). Factor is stable (Weswig, *et al.*, 1946). More active LF and less active SF are both present in fern (Fujita, 1954).
fern labile factor (LF)	No effect	
fern stable factor (SF)	Nervous disorders; death in 24 hours in 40mg% concentration.	
desthiobiotin	No evidence of anorexia; decreased growth or blue slime-patch disease, as in trout. In axenic culture, poor growth, anorexia, darker body coloration, ataxia.	Growth factor in yeast (*Saccharomyces cerevisae*) and antibiotin factor for *Lactobacillus casei* (duVigneaud, 1942); no effect on higher organisms.
deoxypridoxine	Nervous disorders; ataxia; anorexia; decreased growth; rapid and gasping breathing; flexing of opercles; 10% survival at 7 days.	Atrophy and degeneration of spleen and thymus in chicks, rats, dogs and monkeys; microcytic anemia and leucopenia in dogs; dryness of hair, skin scalines , tongue lesions, hyper-irritability and convulsions of epileptic nature in monkeys (Mushett, *et al.*, 1947).
gluco-ascorbic acid	No abnormal indication of growth appetite or mortality in non-axenic fish. Edema, decline in weight in axenic culture.	Growth inhibition, diarrhea, multiple hemorrhages, but no effect on teeth in rats at 10% level in food (Woolley and Krampitz, 1943). Guinea pigs on purified rations and GAA produced disease that was reversed by ascorbic acid (Woolley, 1944). Ascorbic acid at similar 10% level caused similar syndrome in rats except hemorrhages as did 10% of GAA (Banarjee and Elvehjem, 1945).

Discussion

In considering the effects of temperature on the growth of fishes, Brown (1957) pointed out that the slopes of growth curves for fishes may vary considerably according to the degress of detail in the information available. When the growth cycles are "smoothed out" by using annual data for temperate fishes which have annual growth cycles, curves showing lengths or weights plotted against age are generally sigmoid. In the present investigation, such a growth curve was obtained under controlled laboratory conditions. There were, however, short periods when the increase of length was not very great or when there was an actual decrease in weight only. There was never any decrease in the length of the guppy regardless of the experimental procedure employed. The consistent size of the containers also enabled reproductible results which was in agreement with findings of Comfort (1956) who reported specific maximum sizes of fish for each size of container and each level of nutrition. When a fish was transferred from one size container to a larger, or when fish were removed from a tank population, a new plateau was reached.

The thiamine analogs, oxythiamine and pyrithiamine, both elicited polyneuritic-type symptoms in fish, and oxythiamine appeared to be a more powerful antagonist than pyrithiamine. In the lower concentrations of oxythiamine, there was a proportionately larger spread of survival values than in the higher concentrations. More time was required for the analog to take effect. The amount of thiamine in the diet was apparently sufficient to enable the survivors of the first several days to live for periods beyond the 30-day test period. Since an increase of up to 40 μg did not elicit the symptoms any sooner than three days, it is possible that the pre-existing thiamine retarded the onset of symptoms.

Previous studies on mice and rats (Soodak & Cerecedo, 1947), and on chicks (Naber, *et al.*, 1954), have indicated that oxythiamine did not elicit polyneuritic symptoms and was not as powerful an inhibitor as pyrithiamine. The results of this investigation indicated that oxythiamine did elicit polyneuritic-type symptoms in fish and was, in fact, a more powerful antagonist than pyrithiamine. Oxythiamine elicited the characteristic symptoms much sooner and at a much lower concentration than did pyrithiamine.

The differential mode of action of oxythiamine and pyrithiamine in mice and rats has been interpreted to signify that these analogs attack different systems in the tissues (Wooley & Mere-field, 1952). It is possible that the signs of avitaminosis may have been due to an unrecognized function of thiamine not concerned with cocarboxylase or with elevated tissue pyruvate. The different times that oxythiamine and pyrithiamine affected the guppies also indicated their separate role in their blocking of thiamine. If the analogs affected a single metabolic pathway of thiamine, then their action at the minimal effective concentration under identical conditions would have been simultaneous.

The results from the fern extracts indicated that there was a thermostabile substance that was removed by aqueous extraction which was very effective in producing thiamine deficiency symptoms in the guppy. The stable factor was not removed by aqueous extraction. However, cold acetone did remove the stable factor in the filtrate. This stable factor was very active in eliciting deficiency symptoms in the guppy.

The presence of labile and stabile thiamine-destroying factors in bacteria, ferns, crustacea and the viscera of vertebrates presents an interesting situation. Although it has been suggested that thiaminase may be involved in thiamine synthesis, fish require an external source of this vitamin, whereas lower organisms may not require it in the intact molecule. The action or oxythiamine, pyrithiamine and labile and stable fern extracts indicated that there may have been an unrecognized function in synthesis, utilization or otherwise, of thiamine in fishes and other organisms. The mechanisms by which polyneuritic type symptoms have appeared after dietary or analog induced thiamine deficiency in birds and mammals, and now by oxythiamine deficiency in fish, are yet to be described. The natural occurrence of antimetabolites may be correlated with a regulatory or feedback mechanism by which cells may check the synthesis and the useless accumulation of excessive amounts of a metabolite. There is also some evidence that thiaminase may act in bringing together the thiazole and pyrimidine portions of thiamine (Fujita, 1954). Rogers (1962) points out that interest in antithiamines has fluctuated a great deal during the two decades since pyrithiamine was first synthesized. The fundamental aspects of thiamine biochemistry have been greatly clarified during the last three or four years, and more precise and thoughtful studies of antithiamines should thereby be encouraged. A second stimulus may be expected from the area of nervous system biochemistry, since thiamine plays an important but undefined role there. Undoubtedly the thiamine antagonists will aid in its solution.

The biotin analog desthiobiotin has shown competitive inhibition in some microorganisms while it can be synthesized by others (duVigneaud, 1942). This analog has been found to have no activity in vertebrates under typical non-axenic conditions. However, the ability of desthiobiotin to act as a biotin antimetabolite in guppies under axenic conditions was apparently associated with the absence of microorganisms. Phillips, *et al.,* (1950) found that in trout raised on a diet that caused blue slime-patch disease, the younger fish were much more sensitive to the absence of biotin, since they required greater amounts of the vitamin. Trout that survived for a period of four to eight weeks appeared to recover, since there was apparently enough biotin available for their decreased requirements. A similar condition appeared to be present in this investigation. The newborn fish under axenic conditions were much more susceptible to the analog than were fish of eight weeks of age or over.

Pyridoxine or vitamin B_6 has been shown to be dependent on protein intake, and, under circumstances of pyridoxine deficiency in rats, various aspects of protein synthesis were impaired. Deoxypyridoxine, the active analog of pyridoxine, was found to inhibt growth in the guppy because of its possible interference with protein synthesis. Increasing concentrations of antimetabolite elicited a proportionately greater decrease of growth which indicated that the effects of deoxypyridoxine were truly antimetabolic and not toxic.

The literature on the effect of analog induced vitamin C deficiency on various animals is inconsistent. It has been generally known that only the guinea pig, monkey and man could be induced to show signs of vitamin C deficiency by employing an ascorbic acid free diet. In the field of fish nutrition, McLaren, *et. al.,* (1947) reported dietary deficiency effects, while Wolf (1951) and Halver (1953) reported no effects on trout and salmon. These contradictory reports are probably due to the synthesis of ascorbic acid by the intestinal flora. The guppies that were raised non-axenically showed normal growth even though they were subjected to the maximum analog concentration. This was apparently possible since there may have been sufficient ascorbic acid synthesized by the intestinal flora. Interestingly enough, antibiotics, such as aureomycin, decrease the growth rate of the guppy (Berke, Silver & Kupperman, 1953). The present demonstration in which glucoascorbic acid showed activity in the guppy only under axenic conditions indicated that it could have acted as an ascorbic acid antimetabolite in the absence of any bacterial flora.

Summary

The vitamin antimetabolites, oxythiamine, pyrithiamine, extracts from the fern *(Pteris aquilina)*, deoxypridoxine, desthiobiotin and glucoascorbic acid were tested on the guppy *(Lebistes reticulatus)* in non-axenic and axenic conditions. A characteristic growth pattern, as indicated by the lower segment of a sigmoid-type curve, in respect to weight and length, was demonstrated from the time of birth to 12 weeks of age. Oxythiamine was a more powerful thiamine antagonist than pyrithiamine, and both analogs produced polyneuritic-type symptoms. Atypical comparative activity of these substances suggests a different utilization or alternate reaction pathway for thiamine. An aqueous extracted thermolabile and acetone extracted thermostable antithiamine from fern showed reversible thiamine inhibition. Natural antimetabolites may act in the synthesis or in the elimination of excessive amounts of a metabolite. Deoxypyridoxine acted as a pyridoxine inhibitor. Desthiobiotin and glucoascorbic acid were not active antagonists under non axenic conditions. Under axenic conditions, these analogs were active antimetabolites indicating that microorganisms are involved in the synthesis of their respective metabolites. Reversal was demonstrated in all active analogs.

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